Immunological Studies on Pancreatic Phospholipase A₂

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Phospholipase A₂ (EC 3.1.1.4) catalyzes the specific hydrolysis of the ester bond at the C₂₂ position of 1,2-diacyl-sn-phosphoglycerides (1). Ca²⁺ is required as an absolute cofactor.

In the present paper, the microcomplement fixation test (22) appeared to be the most sensitive way to detect (small) conformational differences (23–25) between various porcine phospholipases A₂ and their zymogens and several NH₂-terminal modified enzyme analogs.

It is known that intact γ-globulins cannot be used in kinetic inhibition studies because of their precipitating properties. Monovalent Fab fragments do not show these drawbacks and are suitable for kinetic studies (26–28) without any loss of immunological activity. Making use of the different affinities of the Fab fragments of different antibodies toward NH₂-terminal modified enzyme analogs, a first separation of those fragments was achieved as judged by their different inhibitory properties toward micellar binding and monomeric substrate binding. Also, the calculation of the number of antigenic determinants was more reliable using Fab fragments (19, 29).

MATERIALS AND METHODS

Antigens—Pig (30), horse (31), cow, and sheep (32) (pro)phospholipase A₂ were prepared as previously described. Protein concentrations were determined from the absorbance at 280 nm, using A₁₇₅ of 13.0 for the pig, cow, and sheep enzymes (17, 32) and 12.3 for their zymogens, whereas the values of 12.3 and 11.8 were used for the horse enzyme and zymogen, respectively (31). Snake venom phospholipase A₁ (Notechis scutatus II) was a gift from Dr. D. Eaker, University of Uppsala, Sweden. NH₂ terminally modified ε-amidated phospholipase A₁ analogs were prepared as described before (14). All proteins were checked for purity using polyacrylamide gel electrophoresis.

Immobilization—New Zealand white rabbits (2 kg) received initial doses of 1 mg of pig, horse, cow, and sheep (pro)phospholipase A₂ emulsified in complete Freund’s adjuvant. Injections were given subcutaneously in the back and between the toes. After 1 month, booster injections were given. One week after each booster injection, 20 ml of blood were collected from the ear vein; immune response was monitored by a microcomplement fixation assay (22). Immunization was continued until the maximal level of antibody titer was achieved, usually after about 6 months. At that time, blood was obtained by heart puncture.

Immobilization of Antigens—Some of the antigens, like pig pro-phospholipase A₂, ε-amidated phospholipase, and des-(Ala₁–Arg₇) ε-amidated phospholipase were coupled to ACH-Sepharose (Pharmacia) according to the manufacturer’s instructions. Each coupling
was performed with 25 to 50 mg of enzyme/g of Sepharose. After coupling of the pro-phospholipase, part of it was converted into phospholipase-Sepharose by limited digestion with trypsin (Serva), resulting in the release of the activation peptide and exposure of the free α-NH₂ group of Ala. This roundabout way guarantees the generation of the native conformation of the NH*-terminal region.

**Microcomplement Fixation**—A quantitative microcomplement fixation assay was performed as previously described (22). Before diluting the antigen (protein of interest), complement was first inactivated by heating for 30 min at 56°C. Antiserum pools were made by mixing the individual antisera in inverse proportion to their titers (23). “Immunological distance” has been defined previously (23, 24).

**Preparation of Phospholipase-specific Fab Fragments**—Anti-pig phospholipase γ-globulins were digested with papain according to Porter (35) with some modifications (27). The digestion mixture contained 100 mg of γ-globulin in 10 ml of 0.1 M phosphate buffer, pH 7.0, 2 mM EDTA, 10 mM cysteine, and 1.5 mg of papain (Sigma). After 16 h at 37°C, the mixture was dialyzed at 4°C against 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4. After centrifugation to remove small amounts of insoluble protein, the clear solution was applied to the phospholipase-Sepharose column (the same as previously used). Under those conditions, only Fab fragments and intact γ-globulins are bound. From here, the same procedure was followed as described for the γ-globulins. After concentration the Fab fragments were freed from remaining γ-globulin by passage through a Sephadex G-100 column (1 x 100 cm) the crude γ-globulin fraction was applied. After the respective antisera up, ah, ac, and as. Experimental conditions: 2% agarose A in 0.15 M NaCl and 20 mM Tris-HCl, pH 7.4, was poured in 4-cm Petri dishes (1-mm thick). Cups with sections of 2 mm and mutual distances of 7 to 10 mm were filled with 2 μl (1 μg) of the antigen and 2 μl of the antiserum. After 16 h of incubation at room temperature, the agar was washed with cold buffer containing 0.15 M NaCl and 20 mM Tris-HCl, pH 7.4. Subsequently, the agar was stained with 1% Amido black in 7% acetic acid for 5 min and destained in 7% acetic acid for several hours.

**RESULTS**

**Comparison of the Isoenzymes**—Pancreatic pig, cow, horse, and sheep phospholipases show considerable differences with respect to substrate and Ca²⁺ binding (38). These differences are also detected by immunological methods. As shown in Fig. 1, only cow and sheep phospholipases give precipitin lines of complete identity to both antisera. Horse phospholipase only partially cross-reacts with pig phospholipase using anti-horse phospholipase serum, whereas pig phospholipase shows a partial cross-reaction with horse, cow, and sheep phospholipase toward anti-pig phospholipase serum. The microcomplement fixation test provides more quantitative information. First, some parameters have to be defined (24). The log titer difference or the index of dissimilarity is the experimentally determined degree to which the antiserum concentration must be raised in order to give the same complement fixation for a heterologous antigen with respect to that produced by the homologous antigen. The immunological distance, being 100 times the log titer difference, can be related to the percentage of amino acid sequence difference. Fig. 2 shows this relationship for horse, cow, and pig phospholipase A₂ and their re-

![Fig. 1. Ouchterlony double immunodiffusion analysis of pig (P), horse (H), cow (C), and sheep (S) phospholipase A₁ against their respective antisera ap, ah, ac, and as. Experimental conditions: 2% agarose A in 0.15 M NaCl and 20 mM Tris-HCl, pH 7.4, was poured in 4-cm Petri dishes (1-mm thick). Cups with sections of 2 mm and mutual distances of 7 to 10 mm were filled with 2 μl (1 μg) of the antigen and 2 μl of the antiserum. After 16 h of incubation at room temperature, the agar was washed with cold buffer containing 0.15 M NaCl and 20 mM Tris-HCl, pH 7.4. Subsequently, the agar was stained with 1% Amido black in 7% acetic acid for 5 min and destained in 7% acetic acid for several hours.](http://www.jbc.org/)

![Fig. 2. Dependence of the immunological distance on the percentage of amino acid sequence difference among pig, cow, and horse pancreatic phospholipases. The immunological distance is defined as 100 x log titer difference (23, 24). The line represents an empirical relationship proposed for bacterial azurins (23) and bird lysozymes (24). , anti-cow phospholipase serum against pig phospholipase; , anti-pig phospholipase serum against cow phospholipase; , anti-horse phospholipase serum against pig phospholipase; , anti-pig phospholipase serum against horse phospholipase; , anti-horse phospholipase serum against cow phospholipase; , anti-cow phospholipase serum against horse phospholipase.](http://www.jbc.org/)
Immunological Studies on Pancreatic Phospholipase A

The Immunological Difference of Porcine Phospholipase and Its Zymogen—On tryptic activation of pancreatic porcine pro-phospholipase A₂, the NH₂-terminal heptapeptide: <Glu-Gly-Ile-Ser-Ser-Arg is split off, resulting in the formation of the specific conformation of the NH₂-terminal region of the active enzyme.

Ouchterlony’s immunodiffusion did not discriminate between the enzyme and its zymogen since a complete cross-reaction toward antipolphospholipase serum was observed. However, the complement fixation assay detects a considerable difference as can be seen from Fig. 3. The lower curve clearly reflects the lack of that specific conformation in the zymogen.

In order to investigate to what extent the presumed formation of the salt bridge between Ala₁ and an internal carboxylate would contribute to this immunological difference, a comparison of NH₂ terminally modified analogs was carried out as described in the following paragraph.

Comparison of NH₂ Terminally Modified Enzyme Analogs—Based on the NH₂-terminal modification procedure of porcine ε-amidinated phospholipase A₂ (AMPA) as previously described (14), several AMPA analogs could be compared using the microcomplement fixation assay as can be seen in Fig. 4. In this system, AMPA is defined as the semihomologous antigen. In fact, the positively charged ε-amidated lysines cause only very minor conformational changes as judged by complement fixation (80% with respect to native phospholipase). Substituting L-Ala₁ with D-Ala is reflected by some decrease of complement fixation as shown in Fig. 4. This minor substitution is sufficient to prevent the specific conformation of the NH₂ terminus, and as a result, micelle binding cannot occur (14). Obviously, the difference between d-Ala₁-AMPA and L-Ala₁-AMPA is less than the difference between phospholipase and its zymogen.

Therefore, in addition to the formation of the salt bridge of Ala₁ in phospholipase A₂ or AMPA some other NH₂-terminal conformational change must occur. Successive removal of Ala₁, Leu₉, and Trp₁ shows a substantial decrease of complement fixation. Tryptic digestion of AMPA yields a hexapeptide, Ala₁-Arg₆, and the remaining protein des-(Ala₁-Arg₆)AMPA. As can be seen, the complement fixation of this protein is dramatically decreased. These results provide additional evidence that besides the presumed Ala₁ salt bridge, the other NH₂-terminal residues contribute to the ultimate conformation of AMPA or phospholipase. Moreover, it indicates that at least the sequence Ala₁-Arg₆ may be part of an antigenic determinant.

The Number of Antigenic Determinants of Phospholipase A₂—The conventional quantitative (radio)immunoprecipitation with either pure ¹²⁵I-antiphospholipase γ-globulin or ¹²⁵I-phospholipase gave rise to misinterpretation of the maximum number of antibodies that can simultaneously be bound by phospholipase because no integral number could be calculated. Possibly the existence of nonprecipitating antibodies as reported by some authors (34) may be the cause.

Monovalent Fₐb fragments appeared to be more suitable for this purpose. The procedure is as follows. Fixed amounts of Fₐb fragments (1.2 nmol) were mixed with increasing amounts of ¹²⁵I-labeled phospholipase A₂ (0.18 to 1.1 nmol) in 200 μl of buffer containing 50 ml of Tris, 0.15 M NaCl, and 0.1% bovine serum albumin. After incubation for 30 min at 37°C the samples were applied to a Sephadex column (1 x 100 cm) and eluted with the same buffer.

Fig. 5 shows the elution patterns following the various incubations. The total amounts of radioactivity under the second peak, representing the free antigen, were plotted against the molar ratio of antigen to Fₐb (Fig. 6). Extrapolation

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**Fig. 3.** Microcomplement fixation curves of pig phospholipase A₂ ( ) and its zymogen ( ) against anti-pig phospholipase serum (1:2000).

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**Fig. 4.** Microcomplement fixation curves of several NH₂ terminally modified ε-amidated pig phospholipase A₂ analogs against anti-pig phospholipase serum (1:2000). 

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The abbreviations used are: AMPA, ε-amidated phospholipase A₂; di-C₈PC, 1,2-di-octanoyl-sn-glycero-3-phosphocholine.

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clearly yields a molar saturation ratio of 3 $F_{ab}$/phospholipase. Moreover, the elution volume of the first peak in Fig. 5A, representing the antigen saturated with $F_{ab}$ fragments ($M = 164,000$), is almost equal to that of a sample of $^{125}$I-$\gamma$-globulin ($M = 150,000$), indicating that the antigen can be saturated with three $F_{ab}$ fragments.

Isolation of $F_{ab}$ Fragments with Different Properties—Making use of the indications that the NH$_2$-terminal sequence Ala$_1$-Arg$_e$ may be a part of an antigenic determinant, an attempt was made to isolate the $F_{ab}$ fragments directed toward this determinant employing a series of immunoabsorbent columns as shown in Scheme 1.

To a column (0.8 x 4 cm) containing des-(Ala$_1$-Arg$_e$) AMPA-Sepharose was applied $60$ mg of $F_{ab}$ fragments; $66$% (40 mg) was bound to the immunoabsorbent. Elution of this fraction, so-called $F_{ab}^{NH_2}$-terminal, was achieved as described under “Materials and Methods.” Apparently, this $F_{ab}$ fraction is directed toward the common determinants of both phospholipase and des-(Ala$_1$-Arg$_e$) AMPA.

The unbound $F_{ab}$ fraction was then applied to an AMPA-Sepharose column (0.8 x 4 cm) and another 28% (12 mg) of the original $F_{ab}$ fragments was bound, so-called $F_{ab}^{NH_2}$-terminal. In this case, AMPA was coupled to Sepharose via the only free NH$_2$ group, that of Ala$_1$. Apparently this $F_{ab}$ fraction is directed toward a single antigenic determinant, of which Ala$_1$-Arg$_e$ is a major part, whereas the “Ala$_1$-salt bridge” is not necessary.

Finally the remaining $F_{ab}$ fraction was submitted to a phospholipase-Sepharose column. Here, Ala$_1$ was free to form the salt bridge, because coupling of the antigen had been achieved via the lysine e-NH$_2$ group(s) as outlined under “Materials and Methods.” The final $F_{ab}$ fraction that is bound ($10\% = 6$ mg) is called $F_{ab}^{III}$, and is obviously directed to an antigenic site of phospholipase in which the Ala$_1$ salt bridge conformation is the most important determinant. These three $F_{ab}$ fractions were tested for their inhibitory or protective properties toward micelle binding and active center inactivation, respectively.

Protective Effect of the $F_{ab}$ Fractions toward Active Site Inhibition—As previously shown (18), His$_{al}$ is an active site residue of pancreatic phospholipase A$_2$. Alkylating this residue results in a total loss of monomeric substrate and Ca$^{2+}$ binding, whereas micelle binding remains unaltered. Monomeric substrate analogs and Ca$^{2+}$ ions show a considerable protection against this inactivation.

It was of interest to examine the possible differences between the various $F_{ab}$ fractions upon active site blocking by 1-bromo-2-octanone.

As can be seen from Fig. 7, the $F_{ab}^{NH_2}$-terminal and $F_{ab}^{III}$ do not protect against inactivation, whereas the $F_{ab}^{NH_2}$-terminal fraction does so at increasing concentrations. Apparently, the first two $F_{ab}$ fractions do not block the active site. However, they strongly inhibit micelle binding, as will be shown in the next paragraph.

Inhibition of Micellar Binding by the $F_{ab}$ Fractions—Synthetic 1,2-dioctanoyl-sn-glycero-3-phosphocholine (dilPC) is a suitable substrate to study micellar binding of pancreatic phospholipase A$_2$ (3).

The influence of the different $F_{ab}$ fractions upon micelle binding was examined using enzyme to $F_{ab}$ molar ratios identical with those described in the foregoing paragraph.

In this case, phospholipase activity was determined using 1.7 to 11 mm of the substrate in 2 ml of 0.1 M NaCl and 10 mM CaCl$_2$ equilibrated at 48°C and pH 6.0. The liberated fatty acids were automatically titrated using 4 mM NaOH. The activities are expressed in micromoles·min$^{-1}$·mg$^{-1}$ and have been analyzed by Lineweaver-Burk plotting (39).

As can be seen from Fig. 8, the $F_{ab}^{NH_2}$-terminal fragments cause a mixed type of competitive and noncompetitive inhibition behavior. Graphical analysis (40) yield after some rearrangements for each $F_{ab}$ concentration:

$$
\frac{1}{K_{app}} = \frac{1}{K'} \left( 1 + \frac{[E]}{K} \right)
$$

\textit{ANTI PORCINE PHOSPHOLIPASE A$_2$ $F_{ab}$ FRAGMENTS}

\textit{AMPA-SEPHAROSE}

\textit{AMP A-SEPHAROSE}

$^{20}$%$^{15}$ bound = $^{34}$% bound

$^{10}$%$^{15}$ bound = $^{14}$% bound

$^{0}$%$^{15}$ bound = $^{4}$% bound

\textbf{SCHEME 1. Separation of anti-pig phospholipase A$_2$ ($PA_2$) $F_{ab}$ fragments into differently defined fractions.}
FIG. 7. Protecting effect of F, fragments on the inactivation of pig phospholipase A2 by 1-bromo-2-octanone. Experimental conditions: 2-ml samples containing 3.5 nmol of pig phospholipase and increasing amounts of the different F, fractions (3.5 to 23.5 nmol) in 0.1 M cacodylate/HCl buffer, pH 6.0, and 0.1% bovine serum albumin were first incubated for 30 min at 37°C. Samples (200-ml) were then taken for enzymatic activity in the egg yolk test. These starting activities are referred to as 100% values during inactivation. Then, 20 " of 1-bromo-2-octanone in 96% ethanol was added to the samples and incubation at 30°C was carried on while continuously stirring. At certain time intervals, 200 ml were then taken for the enzymatic assay. The log of the percentage of remaining activity was plotted against the inactivation time. ●-●, without F,; O-O, molar ratio of F, to phospholipase = 8; ▼-▼, molar ratio of F, to phospholipase = 3.3; △-△, molar ratio of F, to phospholipase = 6.7; ▼-▼, molar ratio of F, to phospholipase = 13.4.

FIG. 8. Lineweaver-Burk plots of the inhibition of phospholipase A2 activity toward 1,2-dioctanoyl-sn-glycero-3-phosphocholine hydrolysis by pig phospholipase A2 at 48°C and pH 6.0.

Some properties of the three anti pig phospholipase A2 fractions

<table>
<thead>
<tr>
<th>% Remaining Vmax&lt;sup&gt;a&lt;/sup&gt; at molar ratio Fα&lt;sub&gt;α&lt;/sub&gt;:enzyme</th>
<th>10%</th>
<th>90%</th>
<th>55%</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;s&lt;/sub&gt;</td>
<td>1.10&lt;sup&gt;-9&lt;/sup&gt; M</td>
<td>14.10&lt;sup&gt;-9&lt;/sup&gt; M</td>
<td>9.10&lt;sup&gt;-9&lt;/sup&gt; M</td>
</tr>
<tr>
<td>Type of inhibition</td>
<td>Mixed</td>
<td>Mixed</td>
<td>Noncompetitive</td>
</tr>
</tbody>
</table>

<sup>a</sup>V<sub>max</sub> is defined as the maximal velocity of 1,2-dioctanoyl-sn-glycero-3-phosphocholine hydrolysis by pig phospholipase A2 at 48°C and pH 6.0.

<sup>b</sup>Determined as the protecting effect of the F, fractions on the inactivation of pig phospholipase A2 by 1-bromo-2-octanone.

In this formula, K<sub>app</sub> and K<sub>s</sub> are defined in Fig. 8, whereas "<sub>i</sub> stands for the free [F<sub>α</sub>] minus enzyme-bound [F<sub>α</sub>]. Since enzyme and F<sub>α</sub> have very high affinities and the F<sub>α</sub> to enzyme molar ratio varies only between 1 to 4, the amount of enzyme-bound F<sub>α</sub> cannot be neglected. Considering this amount to be unknown, but rather constant throughout the F<sub>α</sub> concentration range used, Formula 1 can be applied to each F<sub>α</sub> concentration. The three possible combinations of Formula 1 yield a value of K<sub>s</sub> = (1.0 ± 0.2)·10<sup>-9</sup> M. The same analytical procedure was followed for the other F<sub>α</sub> fractions yielding K<sub>s</sub> values of (9 ± 1)·10<sup>-9</sup> M and (14 ± 2)·10<sup>-9</sup> M for F<sub>α</sub> and F<sub>α</sub><sub>terminal</sub>, respectively.

As can be seen from Fig. 8, the V<sub>max</sub> values are influenced differently. Fig. 9 shows the percentage of remaining V<sub>max</sub> calculated from the Lineweaver-Burk plots, as a function of the F<sub>α</sub> to enzyme molar ratio. Table I summarizes the different properties of the free F<sub>α</sub> fractions, which will be discussed.

DISCUSSION

The Difference between the Various Phospholipases—The results obtained with the immunological comparison of the phospholipases A<sub>α</sub> from horse, pig, cow, and sheep pancreas indicate a generally moderate rate of molecular evolution of these enzymes. The considerable immunological and kinetic differences between the horse and cow enzymes (specific activity in the egg yolk test, 2000 and 70 units/mg, respectively) leads to the question: what part of the enzyme molecule is responsible for these differences? As the catalytic center must remain fairly unaltered during molecular evolution, another function of phospholipase A<sub>α</sub>, the micelle binding, has to be considered. It is obvious that this question also requires a physiological approach, because one can imagine that the phospholipid metabolism and composition show substantial differences between the various animals. This results in the presentation of different substrate dispersions to the relevant enzyme. Up till now, we do not know to what extent these factors can contribute to the activity differences observed.
Recently the three-dimensional structure of bovine phospholipase A₂ has been elucidated (41). In combination with enzyme-hybrid experiments and amino acid modification experiments which are in process now, we will be able to study this question in a more direct way.

The Antigenic Determinants—It has been clearly demonstrated that from the possible number of antigenic sites of phospholipase A₂ only three sites can simultaneously be occupied by antibody. This number is equal to that which has been found for proteins with similar molecular weight, like horse cytochrome c (42) and pancreatic ribonuclease (42). It is consistent with the idea that the maximum number of antibodies that can simultaneously be bound by a globular protein is proportional to its available surface (43). However, this number does not take into account the possibility of heterogeneity within the antibody population, which is dependent on the conformational state of the antigen during the immunization course and the immune response of the individual test object.

Let us consider this possibility to be present in our case. During the immunization, phospholipase A₂ might exist in a conformational equilibrium, of which one form has Ala in the NH₂-terminal bridge and the other form has Ala₁₀ free. Antibodies may be elicited against both conformations. This could explain the existence of F₁ and F₁₀₉-terminale representing 10 and 20% of the total F₁₀ population, respectively. The remaining 66% F₁₀₉-terminal fraction may then consist of other members of the phospholipase-specific F₁₀ family, one or more of which influences the catalytic center and the micellar binding site.

The fact that F₁₀₉-terminale and F₁₀ inhibit micellar binding, but not the catalytic center provides additional evidence that the monomer-binding center is located at a distinct position with respect to the micellar binding site.

The intriguing question remains then: how is the monomer phospholipid transported from the micelle to the catalytic site? One of the possibilities is that the actual micelle-binding site of phospholipase A₂ is larger than only the NH₂-terminal region, thus reducing the real distance between the bound micelle and the monomer binding site. The inhibitory properties of the different Fab fractions toward components, as revealed by the Lineweaver-Burk analysis is most indicative. Our aim is a further separation of the Fab and FabHJhH,-tenna' and FiE inhibit micellar binding, one or more of which influences the catalytic center and the micellar binding site. This hypothesis. In particular, their competitive inhibition of this number does not take into account the possibility of heterogeneity within the antibody population, which is dependent on the conformational state of the antigen during the immunization course and the immune response of the individual test object.

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